Annual of Sofia University "St. Kliment Ohridski"
Faculty of Biology
Book 4 - Scientific Sessions of the Faculty of Biology
2020, volume 105, pp. 69-75
Youth Scientific Conference "Kliment's Days", Sofia 2019

THE ASSOCIATION BETWEEN THE PRESENCE OF P16-POSITIVE CELLS IN HUMAN ENDOMETRIUM DURING THE MID-LUTEAL PHASE AND WOMAN'S AGE

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Keywords: p16 senescent cells, human endometrium, age, immunohistochemistry

Abstract: The aim of the present study was to assess the association between the abundance of p16-positive senescent cells in the endometrial compartments during the mid-luteal phase and woman's age. This observational study includes 350 fertile women aged between 24 and 56 years who had an endometrial biopsy during the mid-luteal phase (7 days after LH surge) of the natural cycle. Endometrial biopsies were obtained and tissue sections were stained with an antibody against $p16^{INK4a}$. Statistical analysis was performed by Spearman's correlation test using SPSS v.21 (IBM Corp., Armonk, NY, USA).

No correlation was found between the frequencies of occurrence of p16+ stromal cells and the woman's age ($R=0.056$; $p=0.61$). In contrast, the frequencies of occurrence of p16+ glandular cells and p16+ luminal epithelial cells showed significant correlation with woman's age ($R=0.38$; $p<0.01$ and $R=0.47$; $p<0.01$, respectively).

In conclusion, we found a significant age-dependent increase in the presence of p16-positive senescent cells in the glands and luminal epithelium of the human endometrium. This rise is more evident in the luminal epithelium in comparison to the endometrial glands.

INTRODUCTION

$TP16^{INK4a}$ originates from the *INK4/ARF* locus at human chromosome 9p21.3 and the precise regulation of its expression is crucial for tissue homeostasis and maintaining a balance between tumor suppression and aging in mammalian systems (Sharpless and DePinho, 2007; LaPak and Burd, 2014). This molecule,

known as a tumor suppressor and cell-cycle regulator, is commonly measured in order to explore the level of stress-induced senescence in the human tissues (Serrano et al., 1996) (Collado and Serrano, 2010). $P16^{INK4a}$ is considered also as a robust biomarker of cellular aging in different human tissues (Yang et al., 2008). $P16^{INK4a}$ as a cyclin-dependent kinase inhibitor enforces growth arrest by activation of retinoblastoma protein (Rb) and inactivation of E2F transcription factor (Collado et al., 2007; Rodier and Campisi, 2011). In addition, the expression of $p16^{INK4a}$ and the percentage of p16INK4a positive cells are known to increase with aging in several human and rodent tissues (Kanavaros et al. 2001; Chkhotua et al. 2003; Krishnamurthy et al., 2004). The altered regulation of its expression might contribute to age-associated phenotypes such as type 2 diabetes, atherosclerotic disease, Frailty syndrome, and longevity (Sharpless and DePinho, 2007; Liu et al., 2009; Emanuele et al., 2010). It was also hypothesised that clearance of $p16^{INK4a}$+ cells delays aging associated disorders (Baker et al., 2011). However, data about the endometrial $p16^{INK4a}$+ senescent cells in different compartments and their association with woman's age are still scarce.

The objective of the present study was to assess the association between the abundance of p16-positive senescent cells in the endometrial compartments during the mid-luteal phase and woman's age.

MATERIALS AND METHODS

Subjects and Tissue collection

Three hundred and fifty women were enrolled in the study. All participants provided written informed consent in accordance with the guidelines of the Declaration of Helsinki, 2000. Patients with BMI < 18 kg m^{-2} orBMI ≥ 30 kg m^{-2}, endometriosis, polycystic ovary syndrome, endometrial polyps, myoma, abnormal uterine development and hydrosalpinx were excluded from the study.

The patients underwent a standard endometrial biopsy in spontaneous cycle by pipelle suction between days 20 and 24 of the menstrual cycle based on urinary luteinizing hormone (LH) surge detection (LH +7). LH surge was measured by LH detection kit (Sepal Inc., Boston, MA). The endometrial tissue was submitted to paraffin embedding for immunohistochemical analysis.

Immunohistochemistry

Immunohistochemical analysis was performed on the paraffin-embedded sections by Novolink Polymer Detection System (Leica Biosystems, Wetzlar, Hesse, Germany). Tissue sections were stained with an antibody against $p16^{INK4a}$ (MX007, Master Diagnostica, Granada, Spain).

Each section was deparaffinized in xylene and rehydrated with graded alcohols (70-100%). Subsequently, the sections were placed in 0.01 M citrate buffer (pH 6.0). Next, 3% hydrogen peroxide was applied to block endogenous peroxidase

activity and then the tissue was incubated in 0.4% casein in phosphate-buffered saline (pH 7.6) to reduce nonspecific binding of primary antibody and polymer. After that samples/slides were treated with Novocastra Postprimary Block, containing 10% (v/v) bovine serum in Tris-buffered saline, to enhance penetration of the subsequent polymer reagent. Consequently, poly-HRP anti-mouse/rabbit IgG reagent (NovoLink Polymer) containing 10% (v/v) animal serum in Tris-buffered saline (pH 7.6) was applied to localize the primary antibody, and the reaction product was visualized by incubation with the substrate/chromogen, 3,3′-diaminobenzidine (DAB) prepared from Novocastra DAB Chromogen and NovoLink DAB Substrate Buffer (Polymer), as a brown precipitate. Finally, the sections were counterstained with Novocastra Hematoxylin (0.02%).

Positive staining was identified by brown-colored products in the nucleus and/or in the cytoplasm. Nuclear and cytoplasmic staining in each section was evaluated by the percentage of stained cells.

p16-positive cells were quantified separately in glandular epithelium, luminal epithelium and stromal compartments. The stained and non-stained endometrial stromal cells and luminal epithelial cells were visually counted in three different representative fields for each sample. The number of glands with stained cells was determined by enumeration of 100 glands in each sample. The percentage of stained cells was determined by counting the number of stained and unstained cells in each gland (enumeration of ≥20 glands with stained cells per sample).

Statistical analysis

Statistical Package for the Social Sciences (SPSS) software version 21.0 (SPSS, Inc., Chicago, IL, USA) was used to analyze all data. Spearman's correlation coefficient was used to assess the strength of the linear relationship between the percentage of positively stained cells and women age. $P<0.05$ was considered statistically significant. Data not indicating normal distribution were shown as a median (min-max) value.

RESULTS AND DISCUSSION

The expression of p16 was observed in normal endometrial stroma and the endo¬metrial glandular and luminal epithelial cells (Figure 1). The senescent biomarker p16 was positively stained in particular single cells in the endometrial surface epithelia, glands and stroma. The p16 immunoreactivity occurred in 0.7% (0 ÷ 4.7%) of the stromal epithelial cells. The overall percentage of positive p16 staining of the glandular and luminal epithelial cells was significantly higher within a relatively wide range – 8.0% (0 ÷ 51.2%) and 29.1% (10.1 ÷ 90%), respectively.

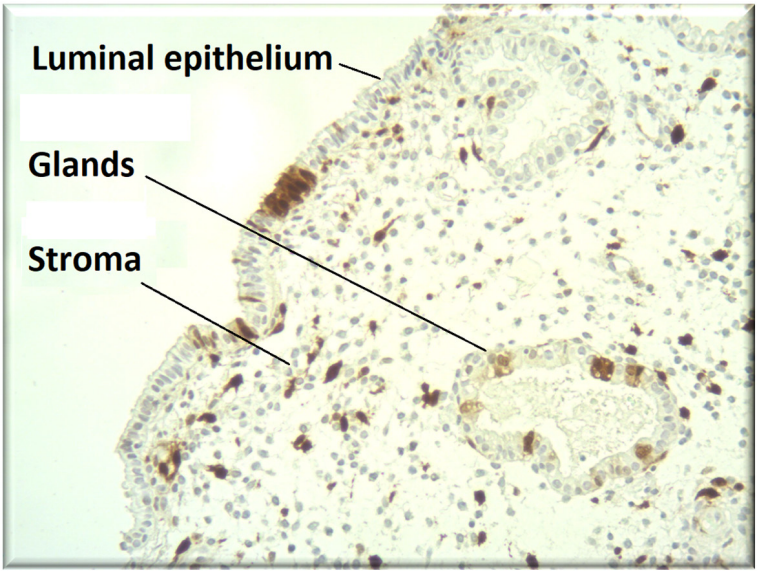

Fig. 1 View of the human endometrium with p16^{INK4a} positively stained cells in the stroma, glands and luminal epithelium (400x magnification).

Correlation analysis revealed significant positive correlation between patient's age and the percentage of p16-stained cells in endometrial glands and luminal epithelium (R=0.38; p<0.01 and R=0.47; p<0.01, respectively) (Fig. 2). On the contrary, the percentage of immunostained cells against p16 in endometrial stroma did not show significant correlation with age (R=0.056; p=0.61) (Table 1).

Table 1 Correlation coefficient (R) between women age and the percentage of immunoreactive cells for the studied senescent biomarker p16^{INK4a} in endometrial luminal epithelium, stroma and glands.

	p16 ^{INK4a}					
	Endometrial stroma		**Endometrial glands**		**Endometrial luminal epithelium**	
	R coefficient	**p-Value**	**R coefficient**	**p-Value**	**R coefficient**	**p-Value**
Women's Age	0,056	0,61	0.38*	<0.01	0.47*	<0.01

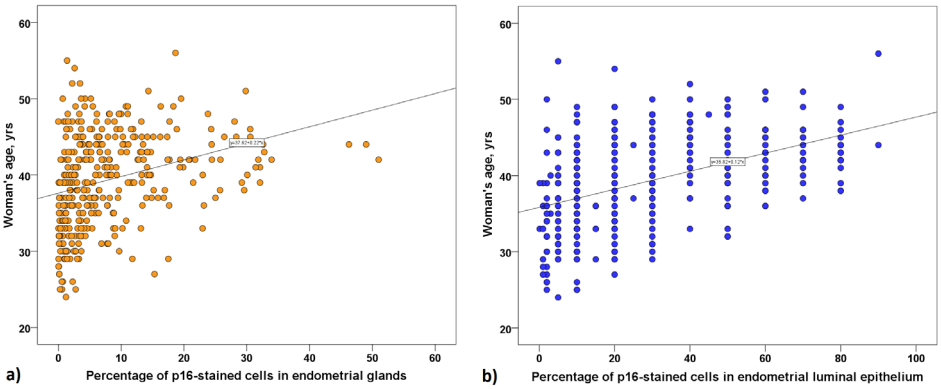

Fig. 2 Scatter plot with line of best fit showing a positive correlation between the patient's age and the percentage of p16-stained cells in (a) human endometrial glands and (b) luminal epithelium (n=350)

The abundance and endometrial tissue distribution of p16+ cells is changing temporally during the menstrual cycle and has a specific quantitative characteristics in the mid-luteal phase. Recent study has shown that p16-positive senescent cells in the mid-luteal phase are involved in endometrial receptivity and in the acute cellular remodeling during the implantation event (Brighton et al., 2017). Our research was focused on this crucial for the implantation process temporal point and in order to be comparable, all biopsy samples were taken seven days after the LH surge.

The obtained results about the percentage of $p16^{INK4A}$+ cells in different compartments of human endometrium during the mid-luteal phase confirmed those from the previous study (Brighton et al., 2017). The registered percentage of senescent cells in the studied endometrial stroma, glands and luminal epithelium was in the same range as described by Brighton et al. The relative abundance of p16 positive cells was also several times higher in the luminal epithelium compared to the glandular compartment. Moreover, we found out that luminal epithelial surface where the first contact with the embryo is made, not only has the largest percentage of $p16^{INK4A}$+ cells, but also the strongest association with women's age.

Our previous study revealed that p21+ as another senescent biomarker in human endometrium had also a strong association with age (Parvanov et al., 2019). The observed correlation between patient's age and the percentage of p21-stained cells in endometrial stroma and glands (R=0.53; p=0.002 and R=0.54; p=0.001, respectively) was slightly lower, but similar compared to the observed in the present study correlation between women's age and p16+ cells in glands and luminal epithelium (R=0.38; p<0.01 and R=0.47; p<0.01). However, it is known that telomere shortening mechanism is bound with the age related senescence, which is connected to activation of cyclin-dependent kinase inhibitor

p21 (Choudhury et al., 2007), while $p16^{ink4a}$ is more tightly associated with stress-induced senescence (Robles and Adami, 1998; Mirzayans et al., 2012).

A possible explanation about the different level of correlation between patient's age and the abundance of p16+ cells in specific endometrial compartments is the assumption that the mechanism driving senescence of endometrial epithelial cells may differ from that in stromal cells (Brighton et al., 2017). For example, unlike their stromal counterparts, endometrial epithelial cells express human telomerase reverse transcriptase (hTERT) and exhibit dynamic telomerase activity (Hapangama et al., 2017). In addition, the senescence in endometrial stroma could lead to specific changes that should not be age-related. Acute senescence of a subpopulation of stromal cells upon decidualization triggers a multi-step process leading to transformation of the endometrium into a gestational tissue. There is increasing evidence for the crucial role of stromal senescence in the endometrial remodelling at the time of embryo implantation. It is controlled spatiotemporally by the level of decidual senescence and the efficacy of immune clearance (Brighton et al., 2017).

CONCLUSION

There is a significant age-dependent increase of the presence of p16-positive senescent cells in the glands and luminal epithelium of the human endometrium. This rise is more evident in the luminal epithelium in comparison with the endometrial glands.

AUTHOR CONTRIBUTIONS

D.P., R.G. and G.S. conceived the experiment; D.P., N.V. and R.G. conducted the experiment; D.P., R.G. and D.N. analyzed the results. D.P., R.G. and D.N. wrote the main manuscript text and prepared tables and figures. G.S., D.P., D.N., and R.G. edited the manuscript and made its final revision. All authors critically reviewed and approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

Acknowledgement: This work was supported by Nadezhda Women's Health Hospital, Sofia, Bulgaria.

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